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EFFECT OF NITRATE ON OIL CONTENT AND FATTY ACID COMPOSITION OF *Nannochloropsis* SP. AT EARLY STATIONARY GROWTH PHASE

THYE SAN CHA[1,2,*], FONG LEE NG[2], AHMAD AZIZ[2] AND SAW HONG LOH[3]

[1]*Institute of Marine Biotechnology,* [2]*Department of Biological Sciences, Faculty of Science and Technology,* [3]*Department of Chemical Sciences, Faculty of Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia.*

**Corresponding author: cha_ts@umt.edu.my, aaziz@umt.edu.my, lohsh@umt.edu.my*

Abstract: A study was conducted to investigate the effect of different nitrate concentrations (0.03, 0.05, 0.09, 0.18, 0.34 and 0.66 mM) on oil content and fatty acid composition of *Nannochloropsis* sp. (strain UMT-M3) at early stationary growth phase. Results showed that the biomass production increased approximately 7-folds at 0.34 and 0.66 mM nitrates (p>0.05), which produced between 0.63 to 0.78 g L^{-1} of cells dry weight. The highest oil content of 13.3% (dry weight basis) was obtained at 0.34 mM nitrate. The analysis of oil revealed that palmitic (C16:0), stearic, (C18:0), oleic (C18:1), linoleic (C18:2), gamma-linolenic (C18:3n6) and alpha-linolenic (C18:3n3) acids were the major type of fatty acids detected in all nitrate treatments. Interestingly, the accumulation pattern for C16:0 was completely in reverse of C18:2 and C18:3n3 in nitrate, ranging from 0.03 to 0.18 mM. The most critical regulatory point occurred at 0.05 mM nitrate, where the highest (p<0.05) accumulation of C16:0 (40.6%) was at the expense of C18:2 (11.4%). Surprisingly, the content of C18:1 (31.0 – 35.0%) was unaffected in all nitrate concentrations. These observations lead to the postulation that the activities of genes, such as Δ12- and Δ15-desaturases that regulate the synthesis of C18:2 and C18:3n3 are concerted in actions and are in reverse of palmitoyl-ACP thioesterase gene, which regulated the synthesis of C16:0.

KEYWORDS: Fatty acids, microalgae, *Nannochloropsis*, nitrate, oil

Introduction

Microalgae are photosynthetic organisms which represent a diverse group of eukaryotic organisms which play an important role in aquatic ecosystems (Feng *et al.,* 2005). They form the foundation of most aquatic food chains and webs as primary producers (Moreno-Garrido, 2008). Microalgae are of great potential as an economical source of valuable natural compounds, such as pigments (Dufossé *et al.,* 2005), polysaccharides (Brown *et al*., 1997), polyunsaturated fatty acids (PUFAs) (Wen & Chen, 2003; Feng *et al*., 2005) and bioactive compounds (Leon-Banares *et al.,* 2004). Therefore, they are commercially important in a sustainable food-supply chain. In recent years, the use of microalgae in biotechnology has increased. These organisms are exploited in the food, cosmetic, aquaculture and pharmaceutical industry (Leon-Banares *et al*., 2004). One of the interesting characteristics of microalgae is their rich oil content, some of which could exceed 80% of the dry weight of their biomass, while oil content of 20-50% is common (Spolaore *et al.,* 2006). Algal lipids are composed of glycerol, sugars or bases esterified to saturated or unsaturated fatty acids (12-22 carbons). Among all the fatty acids in microalgae, some fatty acids of the omega-3 and omega-6 families are of particular interest (Spolaore *et al*., 2006).

Nannochloropsis has been widely used as feed in many mariculture systems (Lubzens, 1995) and as an alternative source of pigments of great commercial value, such as chlorophyll a, beta-carotene, violaxanthin and vaucheriaxanthin (Macías-Sánchez *et al.,* 2005). The species is considered one of the most promising photoautorophic producers of eicosapentaenoic acid (EPA) and has been the most commonly-used algae in many mariculture hatcheries (Cheng-

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Wu *et al.,* 2001). However, the total lipid content and fatty acid composition of the species can be affected by nutritional and environmental factors (Wen & Chen, 2003).

Nitrogen limitation or starvation culture medium has been known to strongly influence the metabolism of lipids and fatty acids in various microalgae. Generally, cultivation in low nitrogen concentration medium increases the lipid accumulation in their cells and decreases the total content of PUFAs (Basova *et al.,* 2007). The aim of this study was to investigate the effects of different nitrate concentrations on the oil and fatty acid composition of *Nannochloropsis* sp. (strain UMT-M3) at early stationary growth phase.

Materials and Methods

Algal strain and culture conditions

Nannochloropsis sp. (strain UMT-M3) was obtained from marine microalgae stock culture at Universiti Malaysia Terengganu, Malaysia. The algae cultures were initiated from a single colony taken from the stock agar plate in f/2 medium prepared with natural sea water (5 ppt). The final nitrate concentrations in culture media were 0.03, 0.05, 0.09, 0.18, 0.34 and 0.66 mM, which were prepared by adjusting the sodium nitrate ($NaNO_3$) concentration of the f/2 medium. The nitrate contributed by sea water was determined according to Collos *et al.,* (1999). The initial cell density was standardised at 5×10^5 cells mL^{-1} in 2.5 L culture medium prepared with sea water (5 ppt) in 3 L conical flask. The cultures were maintained at 25 ± 1 °C, 12:12 h light-dark cycles and light intensity of ~80 µmol m^{-2} s^{-1} with constant aeration. The growth of microalgae was monitored by measuring the OD_{600} value of the cultures and cell density was calculated from established calibration curve. The cultures were monitored and harvested at early stationary growth phase when cells growth became plateau. Cells were harvested by flocculation and centrifugation as described by Lee *et al.* (1998). Finally, the cells were dried at 80 °C in the oven until constant weight was obtained. The biomass production (g L^{-1}) was estimated as dried cells weight divided by volumes of culture medium.

Oil extraction and fatty acid analysis

The oil extraction and fatty acid analysis procedures used in this study were as described by Cha *et al.,* (2011). In brief, 0.5 g of dried sample was first hydrolysed with concentrated hydrochloric acid followed by three rounds of hexane extractions. The hexane was removed with the Rotavapor R-210/215 (Buchi) and the oil content was measured gravimetrically and expressed as a dry weight percentage. The extracted oil (50 mg) was esterified using a Lieberg Condenser system by sequentially adding the 0.5 N NaOH (prepared in methanol), boron-trifluoride (in 20% methanol) and n-heptane. After that, the Lieberg Condenser was removed and 15 mL of saturated sodium chloride was added into the mixture and shaken vigorously for 15 s before pouring into a test tube. Finally, the upper layer was transferred into a 1.5 mL vial with addition of sodium sulfate anhydrous to absorb the water. The fatty acid methyl esters (FAMEs) were analysed using gas chromatography with HP88 capillary column (Supelco) and a flame ionisation detector (HP6890, Agilent Technologies, USA) with conditions as previously described (Cha *et al*., 2011). The Supelco 37 Component FAME Mix (Sigma-Aldrich) was used as a reference standard to identify and quantitate the percentage of *cis*- and *trans*-fatty acid isomers of the samples.

Statistical analysis

The effect of nitrate on oil content and fatty acid profiles of *Nannochloropsis* sp. was determined in three replicates and data were checked for normality and equal variances before being analysed statistically by one-way analysis of variance (ANOVA). The significant differences were identified by post-hoc Tukey's honestly significant difference (HSD) test at $p<0.05$.

Results and Discussion

Growth and oil content of Nannochloropsis sp.

Nannochloropsis sp. (UMT-M3) cells were harvested at early stationary growth phase. Generally, cells cultured in low nitrate of 0.09 mM and below showed a relatively slow growth and attained cell density of less than 1×10^7 cells

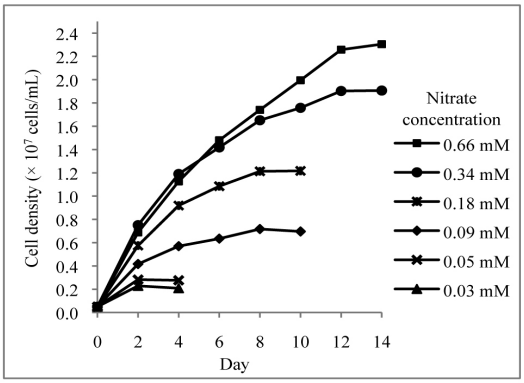

Figure 1. Growth curves of *Nannochloropsis* sp. (UMT-M3) in medium supplemented with different nitrate concentrations. The cells were harvested at early stationary growth phase when cells growth became plateau.

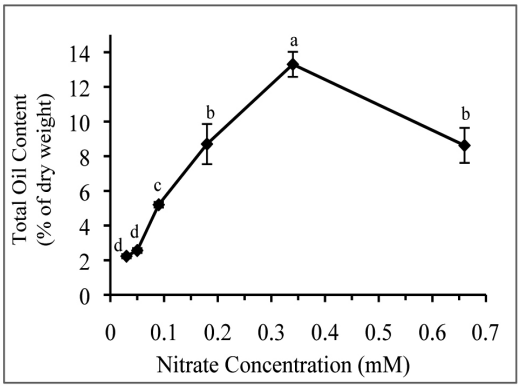

Figure 2. Effect of nitrate on total oil content of *Nannochloropsis* sp. (UMT-M3). Vertical bars represent standard deviation of the mean. Means followed by the same letter are not significantly different according to Tukey's Honesty Significant Difference (HSD) test at p=0.05.

mL^{-1}, while cultivation time taken to reach early stationary growth phase increased with increasing nitrate concentration as shown in Figure 1. The growth in media with 0.03 and 0.05 mM nitrates became plateau in a relatively short period of 4 days. The time taken to reach stationary growth phase showed a leap of 2.5-folds to 10 days when the nitrate concentrations were increased to 0.09 and 0.18 mM. The stationary growth phase was further increased to 14 days in cultures with 0.34 and 0.66 mM nitrates (Figure 1). The cell density increased gradually from 2.08 × 10^6 cells mL^{-1} in medium with 0.03 mM nitrate to highest cell density of 2.30 × 10^7 cells mL^{-1} at 0.66 mM nitrate (Table 1). Hii, *et al.,* (2011) reported that *Nannochloropsis* sp. cultivated in nitrate (0.9 mM) enriched f/2 medium with initial inoculum of 1 × 10^6 cell mL^{-1} achieved stationary growth phase in 8 days, but with lower cell density of 5 × 10^6 cell mL^{-1}. The biomass production was higher (p<0.05) in media with 0.34 and 0.66 mM nitrates, which produced 0.63 and 0.78 g L^{-1} of cells dry weight, respectively. The results were comparable to 0.58 to 0.66 g L^{-1} of biomass produced by *Nannochloropsis oculata* at stationary growth phase as reported by Su *et al.,* (2011) in a single-stage batch cultivation. On the other hand, the biomass production was the lowest (0.1 g L^{-1}) at 0.03 mM nitrate, while there was no significant difference (p>0.05) in nitrate ranging from 0.05 to 0.18 mM, which produced between 0.26 to 0.46 g L^{-1} of cells dry weight (Table 1). The biomass of lower than 0.5 g L^{-1} was also reported for this species cultivated in mixotrophic and heterotrophic conditions (Fang *et al.,* 2004).

Previous studies showed that several microalgae species, such as *Chlorella vulgaris* (Feng *et al.,* 2011b), *Gymnodinium* sp. (Mansour *et al.,* 2003), *Thalassiosira pseudonana* and *Pavlova lutheri* (Tonon *et al.,* 2002) accumulated higher lipid content at stationary growth phase than exponential growth phase. Therefore, the oil content and fatty acid composition of *Nannochloropsis* sp. (UMT-M3) were evaluated at stationary growth phase in this study. Results showed that *Nannochloropsis* sp. cultured in media with low nitrate concentrations (0.03 and 0.05 mM) produced between 2.22 to 2.59% (of dry weight basis) of total oil content (Figure 2). The accumulation of oil was then increased gradually with the increasing nitrate concentration until 0.34 mM, where it achieved the highest (p<0.05) value of 13.3%. However, further increment of nitrate concentration to 0.66 mM caused a significant reduction (p<0.05) in oil production to 8.62%, which is similar to the oil produced at 0.18 mM nitrate (Figure 2). Nitrogen-free or nitrogen-limitation culture conditions were reported to increase the accumulation of storage lipids in *Chlorella* (Illman *et al.,* 2000; Converti *et al.,* 2009), *Nannochloris* sp. (Yamaberi *et al.,* 1998)

Table 1. The biomass, total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids of *Nannochloropsis* sp. (UMT-M3) cultured in different nitrate concentrations.

Nitrate concentration (mM)	Cells dry weight (g L^{-1})	SFA	MUFA	PUFA
0.03	0.10±0.01^{c}	43.93±1.15^{b}	33.17±0.93^{ab}	22.90±1.65^{b}
0.05	0.28±0.10^{b}	48.93±0.99^{c}	33.03±2.58^{ab}	18.00±1.77^{a}
0.09	0.26±0.01^{b}	42.80±1.61^{b}	31.97±1.37^{a}	25.20±1.65^{bc}
0.18	0.46±0.02^{b}	35.60±0.62^{a}	33.50±0.26^{ab}	30.87±0.74^{d}
0.34	0.63±0.04^{a}	34.37±1.05^{a}	35.90±1.00^{b}	29.70±2.00^{d}
0.66	0.78±0.05^{a}	35.13±2.29^{a}	35.87±0.95^{b}	29.03±1.42^{cd}

Means followed by the same letter within the same column are not significantly different according to Tukey's Honesty Significant Difference (HSD) test at p=0.05.

and *Pavlova viridis* (Li *et al.,* 2005). Other studies showed that species such as *Ellipsoidion* sp. (Xu *et al.,* 2001) and *Isochrysis zhangjiangensis* (Feng *et al*., 2011a) were able to accumulate higher oil content in media supplemented with high nitrate concentration of 1.92 and 9 g L^{-1}, respectively. The results of this study indicated that factors other than nitrate became insufficient during the cultures that occurred at 0.66 mM of nitrate, where the accumulation of oil in this species showed a significant decline (Figure 2). Therefore, other factors such as the availability of iron (Liu *et al.,* 2008), organic carbon sources (Taoka *et al.,* 2011) and CO_2 aeration (Chiu *et al.,* 2009) in culture medium, as well as high light intensity (Gordillo *et al*., 1998) may also play significant roles in determine the lipid content of this microalga species.

Fatty acid composition of Nannochloropsis sp. *at early stationary growth phase*

The effect of different nitrate concentrations on fatty acid composition of *Nannochloropsis* sp. strain UMT-M3 was determined at early stationary growth phase when cells growth became plateau (Figure 1). The total of saturated fatty acids (SFAs) was significantly higher (p<0.05) at low nitrate concentrations of 0.03 mM to 0.09 mM, where the production ranged from 42.8% to 48.9% (all percentages for fatty acid composition were of total oil content) as compared to 34.4% to 35.7% obtained at 0.18 to 0.66 mM nitrate concentrations (Table 1). Palmitic acid (C16:0) was the major type of SFA, its content increased (p<0.05) from 35.8% to the highest of 40.6% when nitrate was increased from 0.03 mM to 0.05 mM. Thereafter, the accumulation of C16:0 declined to 28.0% at 0.18 mM nitrate, while further increase of the nitrate concentration to 0.34 mM and 0.66 mM had no effect on C16:0 contents (p>0.05). The synthesis of stearic acid (C18:0), which ranged from 6.0 to 6.7%, was not significantly (p>0.05) affected by nitrate (Figure 3a).

The total monounsaturated fatty acids (MUFAs) production by the species was between 32.0% to 35.9% (Table 1), with oleic acid (C18:1) was the most abundant type of MUFA. The results showed that the cultivation of *Nannochloropsis* sp. at 0.34 mM and 0.66 mM nitrates accumulated higher (approximately 36.0%) C18:1 as compared to 31.0% accumulated at 0.09 mM nitrate (p<0.05) (Figure 3a).

The accumulation of total polyunsaturated fatty acids (PUFAs) in *Nannochloropsis* sp. showed a completely reverse trend as compared to total SFAs at nitrate ranging from 0.03 mM to 0.18 mM (Table 1). The analysis of individual PUFA revealed that linoleic acid (C18:2) was the major component of PUFA, followed by alpha-linolenic acid (C18:3n3) and gamma-linolenic acid (C18:3n6). Interestingly, the species depicted similar accumulation pattern for C18:2 and C18:3n3. The content of C18:2 and C18:3n3 decreased from 14.8% and 4.8% to the lowest of 11.4% and 3.1%, respectively when the nitrate was increased from 0.03 to 0.05 mM. Thereafter, the production recovered until 0.18 mM nitrate, where the cells accumulated 18.7% of C18:2 and 8.1% of C18:3n3, while no further increment was observed at higher nitrates (Figure 3b). On

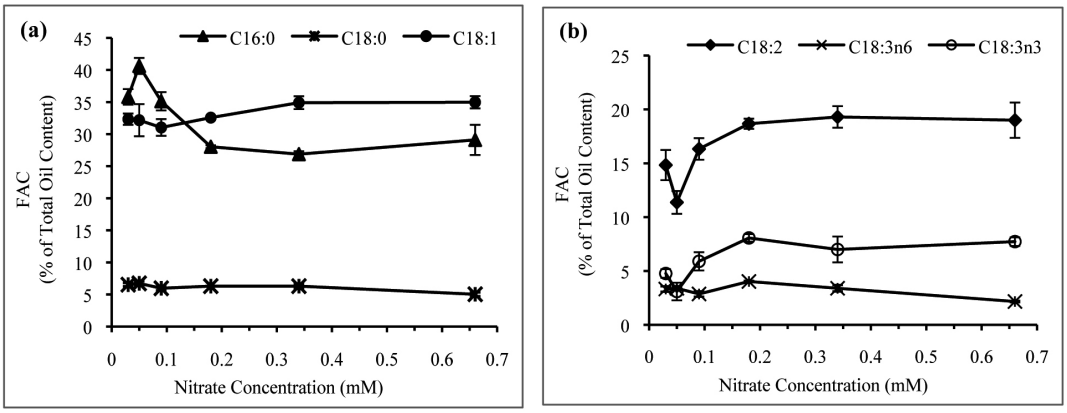

Figure 3: Effect of nitrate on (a) palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and (b) linoleic acid (C18:2), gamma-linolenic acid (C18:3n6) and alpha-linolenic acid (C18:3n3) of *Nannochloropsis* sp. (UMT-M3). FAC: fatty acid composition. Vertical bars represent standard deviation of the mean.

the other hand, gamma-linolenic acid (C18:3n6) production was relatively low (≤ 4%) for this species in all nitrates concentrations. Previous studies had reported that *Nannochloropsis* strains (Fang *et al*., 2004; Su *et al*., 2010) were able to accumulated eicosapentaenoic (C20:5n3) and palmitoleic (C16:1n7) acids. However, the *Nannochloropsis* sp. (strain UMT-M3) used in this study did not produce detectable amount of C20:5n3 and C16:1n7 under the studied conditions (data not shown).

Regulation of fatty acid biosynthesis in response to nitrate

At early stationary growth phase, *Nannochloropsis* sp. displayed drastic changes in the accumulation of C16:0, C18:2 and C18:3n3, which occurred at nitrates ranged from 0.03 mM to 0.18 mM as shown in Figure 3. The accumulation pattern for C16:0 was completely in reverse of C18:2 and C18:3n3. The most interesting regulatory point was at 0.05 mM nitrate, where the highest accumulation of C16:0 (40.6%) was at the expense of C18:2 (11.4%). Furthermore, the drop in the accumulation of C16:0 from 0.05 mM to 0.18 mM nitrates was coupled with the increase of C18:2 and C18:3n3 content. Surprisingly, the content of C18:1; which was the single most abundant MUFA in the *Nannochloropsis* cells appeared unaffected. In contrast, the cultivation of *Parietochloris incise* (Solovchenko *et al.,* 2008), *Botryococcus braunii* (Choi *et al.,* 2010) and *Scenedesmus rubescens* (Lin & Lin, 2011) under nitrogen limitation conditions were reported to accumulate higher C18:1 as compared to C16:0 and C18:3n3. The accumulation of C16:0 and C18:1 in *Ellipsoidion* sp. increased at the expense of PUFAs, in particular the eicosopentaenoic acid (C20:5n3) when cultivated under nitrogen limitation condition (Xu *et al.,* 2001). Thus, it can be concluded that the accumulation of PUFAs, in particular the C18:2 and C18:3n3, were closely linked and was in reverse of the accumulation trend for C16:0 in *Nannochloropsis* sp. in response to nitrate limitations at early stationary growth phase (Figure 3). These observations led to the postulation that the expression of genes, such as Δ12- and Δ15-desaturases that regulate the synthesis of C18:2 and C18:3n3 were concerted in actions and were in reverse of palmitoyl-ACP thioesterase gene, which regulated the release of C16:0-CoA. Previous study showed that concerted expression of Δ12, Δ6 and Δ5-desaturase genes in *Parietochloris incise* cells subjected to nitrogen starvation led to the higher accumulation of arachidonic acid (Iskandarov *et al.,* 2010).

Conclusions

The highest oil production for cultivated *Nannochloropsis* sp. (UMT-M3) was in f/2 medium supplemented with 0.34 mM nitrate. The species also accumulated relatively high

amount of C16:0 (26.9%), C18:1 (34.9%) and C18:2 (19.3%) at 0.34 mM nitrate. However, nitrate concentrations lower than 0.18 mM showed obvious influence on the regulation of fatty acid biosynthesis, in particular the C16:0, C18:2 and C18:3n3. Thus, it would be interesting to investigate the expression of related genes in relation to the accumulation of those fatty acids at nitrate ranging from 0.03 to 0.18 mM in future study.

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